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# A common mechanism for resistance to oxime reactivation of acetylcholinesterase inhibited by organophosphorus compounds

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ABSTRACT

Administration of oxime therapy is currently the standard approach used to reverse the acute toxicity of organophosphorus (OP) compounds, which is usually attributed to OP inhibition of acetylcholinesterase (AChE). Rate constants for reactivation of OP-inhibited AChE by even the best oximes, such as HI-6 and obidoxime, can vary >100-fold between OP-AChE conjugates that are easily reactivated and those that are difficult to reactivate. To gain a better understanding of this oxime specificity problem for future design of improved reactivators, we conducted a QSAR analysis for oxime reactivation of AChE inhibited by OP agents and their analogues. Our objective was to identify common mechanism(s) among OP-AChE conjugates of phosphates, phosphonates and phosphoramidates that result in resistance to oxime reactivation. Our evaluation of oxime reactivation of AChE inhibited by a sarin analogue, *O*-methyl isopropylphosphonofluoridate, or a cyclosarin analogue, *O*-methyl cyclohexylphosphonofluoridate, indicated that AChE inhibited by these analogues was at least 70-fold more difficult to reactivate than AChE inhibited by sarin or cyclosarin. In addition, AChE inhibited by an analogue of tabun (i.e., *O*-ethyl isopropylphosphonofluoridate) was nearly as resistant to reactivation as tabun-inhibited AChE. QSAR analysis of oxime reactivation of AChE inhibited by these OP compounds and others suggested that the presence of both a large substituent (i.e., ⩾the size of dimethylamine) and an alkoxy substituent in the structure of OP compounds is the common feature that results in resistance to oxime reactivation of OP-AChE conjugates whether the OP is a phosphate, phosphonate or phosphoramidate.

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## 1. Introduction

The acute toxicity of organophosphorus (OP) compounds is usually attributed to their inhibition of acetylcholinesterase (AChE; E.C. 3.1.1.7) [1,2]. To mitigate the toxic effects of OP compounds, oximes were developed that were capable of reactivating OP-inhibited AChE [2]. However, reactivation of OP-inhibited AChE by even the best oximes varies significantly for AChE inhibited by different OP agents [3–6]. For example, the mono-pyridinium oxime 2-PAM is effective against human AChE inhibited by VX and sarin, but it is not effective against human AChE inhibited by VR, cyclosarin or tabun [6]. Although bis-pyridinium oximes, such as HI-6 or obidoxime, provide better reactivation against human AChE inhibited by VR and cyclosarin, tabun-inhibited human AChE is poorly reactivated by these oximes [6]. The mechanism of resistance to reactivation for these OP-AChE conjugates is not well defined [7,8], and further clarification is needed to rationally design a reactivator that is effective against a variety of OP agents [9,10].

## 2. Methods

### 2.1. Materials

Sarin, methylsarin, cyclosarin, tabun, VX, *O*-methyl isopropylphosphonofluoridate (sarin analogue), *O*-methyl cyclohexylphosphonofluoridate (cyclosarin analogue) and *O*-ethyl isopropylphosphonofluoridate (tabun analogue) were obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) and were >98% pure as determined by $^{31}$P-nuclear magnetic resonance spectroscopy. Paraoxon, methyl paraoxon and methamidophos were purchased from ChemService (West Chester, PA). HI-6 dichloride and obidoxime dichloride were obtained from the Walter Reed Army Institute of Research (Washington, DC).

Recombinant human AChE was a generous gift from Nageswararao Chilukuri (US Army Medical Research Institute, Aberdeen Proving Ground, MD). This enzyme had been expressed in a Chinese Hamster Ovary cell line that was stably transfected with

*Abbreviations:* AChE, acetylcholinesterase; OP, organophosphorus; QSAR, quantitative structure–activity relationship; VR, *O*-isobutyl methylphosphonofluoridate.

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a full-length cDNA for human AChE. The enzyme was purified by affinity chromatography on a procainamide-Sepharose 4B affinity column to a final specific activity of 345 units/nmol.

### 2.2. Oxime reactivation of OP-inhibited AChE

Oxime reactivation studies were performed with AChE that had been incubated for 30 min with sufficient OP agent to achieve >95% inhibition of AChE activity. Excess OP was removed by passing the OP/AChE mixture through a Sephadex G-25 PD-10 column. Oxime reactivation studies of OP-inhibited AChE (1 nM) were conducted at 25 °C in pH 7.4 phosphate buffer (50 mM) with a range of oxime concentrations from 0.5 to 1000 μM for each oxime. AChE activity was measured at 25 °C by the Ellman method [11] in 50 mM phosphate buffer (pH 7.4) using acetylthiocholine as substrate.

Analysis of the kinetics of oxime reactivation of OP-inhibited AChE was based on the scheme:

$$(EP) + (Ox) \overset{K_D}{\leftrightarrows} (EP \cdot Ox) \overset{k_r}{\rightarrow} (E) + (P - Ox)$$

where (EP) is the phosphylated enzyme, (Ox) is the reactivating oxime, (EP·Ox) is the complex formed by the phosphylated enzyme and oxime, (E) is the reactivated enzyme, (P-Ox) is the phosphylated oxime, $K_D$ is the dissociation constant of the (EP·Ox) complex and $k_r$ is the maximal first-order rate constant for reactivation [3]. The observed first-order rate constant for reactivation ($k_{obs}$) can be described by Michaelis–Menten kinetics where $k_{obs} = k_r\,(Ox)/(K_D + (Ox))$. Accordingly, all oxime reactivation studies were conducted with $(Ox) > (EP)_0$ to establish pseudo-first-order reaction conditions to simplify the determination of kinetic constants. Because $k_{r2} = k_r/K_D$ when oxime concentrations (Ox) are $<<K_D$, the second-order rate constant $k_{r2}$ describes the reactivation of agent-inhibited AChE at the low physiological concentrations of oximes that are typically observed *in vivo*. Therefore, values for $k_{r2}$ were used to compare oxime reactivation rate constants among OP-AChE conjugates.

### 2.3. Analysis of quantitative structure–activity relationships (QSAR)

QSAR for oxime reactivation were identified by analysis of linear free energy relationships between the structure of oximes and OP compounds and the ability of oximes to reactivate OP-inhibited AChE. Multiple linear regression equations were analyzed using SigmaStat for Windows, version 2.03 (Jandel Scientific, San Rafael, CA). Independent variables were considered to make statistically significant contributions to prediction of reactivation when P for the variable was <0.05.

The electronic constants of substituents ($\sigma^{ph}$) determined by Mastryukova and Kabachnik [12] were used to estimate the electronic effects of OP substituents on reactivation. The steric effects of OP substituents were evaluated by using their molecular volumes, which were estimated by the method of Connolly [13]. OP substituents were assigned to either the acyl or choline pockets of AChE based on our model of the orientation of OP structures in the active site of AChE. Our model was based on the generally reported orientation of OP substituents in the crystal structures of OP–AChE conjugates [14–16]. In these 3-D crystal structures the acyl pocket binds small OP substituents through hydrophobic bonding but not hydrogen bonding, while the choline pocket binds larger OP substituents through hydrogen bonding to a hydrogen acceptor, such as an alkoxy substituent.

## 3. Results and discussion

Our initial QSAR analysis was conducted with a data base for oxime reactivation of OP-inhibited human AChE that had been generated by Worek and co-workers [3–5]. This data set contained AChE reactivation data for 43 oxime/phosphonate pairs, 21 oxime/phosphoramidate pairs and 12 oxime/phosphate pairs. The best linear regression equation resulting from multiple regression analysis of oxime reactivation ($k_r$) of OP-inhibited AChE against a variety of structural, electronic and steric parameters was the following:

$$log(k_r) = 4.90 - 0.03V_{acyl} + 0.66(\sigma_{acyl} + \sigma_{chol}) + 0.54P_{ox} - 0.053pK_a$$

where $V_{acyl}$ is the molecular volume of OP substituent in the acyl pocket, $\sigma_{acyl} + \sigma_{chol}$ is the sum of electronic effects of the OP substituents in the acyl and choline pockets, $P_{ox}$ is the position of the oxime group as either ortho or para on a pyridinium ring and $pK_a$ describes the ionization of the oxime group on a pyridinium ring.

This QSAR equation achieved a correlation coefficient ($r$) of 0.85 ($n = 76$). A summary of the statistical results associated with this equation is shown in Table 1. The marginal sum of squares, which is a measure of the reduction in the sum of the squared residuals contributed by each parameter, was used to quantify the contribution of each parameter in predicting $k_r$. The molecular volume of the OP substituent in the acyl pocket ($V_{acyl}$) made the greatest contribution (55% of total explained variation) toward explaining the variation in $k_r$, while the sum of the electronic effects of the OP substituents ($\sigma_{acyl} + \sigma_{chol}$) made a much smaller contribution (14.8%) toward explaining $k_r$.

**Table 1**
Statistical summary of QSAR equation.

| Parameter | Parameter coefficient | Standard error of coefficient | $P$ | Marginal sum of squares | % Contribution to explained variation |
|---|---|---|---|---|---|
| $V_{acyl}$ | −0.031 | 0.003 | <0.01 | 12.82 | 55.1 |
| $\sigma_{acyl} + \sigma^{ch}_{ol}$ | 0.66 | 0.12 | <0.01 | 3.44 | 14.8 |
| $P_{ox}$ | 0.54 | 0.08 | <0.01 | 4.41 | 18.9 |
| $pK_a$ | −0.053 | 0.011 | <0.01 | 2.60 | 11.2 |

Based on our statistical analysis of oxime reactivation of OP-inhibited AChE, we predicted that the presence of a large OP substituent in the acyl pocket of AChE should produce a negative effect on oxime reactivation of OP-inhibited AChE (note the negative sign on the $V_{acyl}$ coefficient in the QSAR equation) in comparison to reactivation of AChE inhibited by an OP with a methyl substituent in the acyl pocket, such as sarin. We also predicted that the molecular volume of an OP substituent in the acyl pocket would have a greater negative effect on reactivation than the electronic donation or withdrawal of the subsituent. Consequently, we predicted that the major impediment to reactivation of AChE inhibited by a phosphoramidate, such as tabun, would be the size of the OP substituent in the acyl pocket and not its electronic properties. In order to test our predictions we compared the reactivation of the OP agents and their analogues shown in Fig. 1.

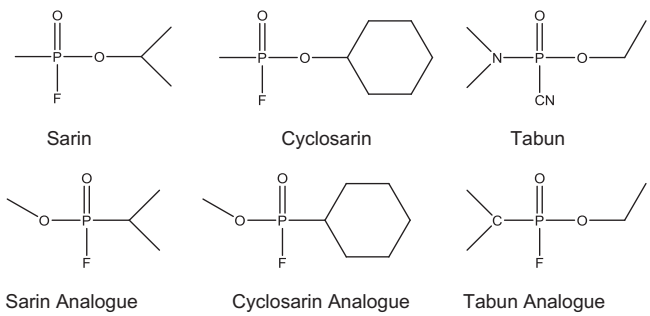


**Fig. 1.** Chemical structures of parent OP agents and analogues.

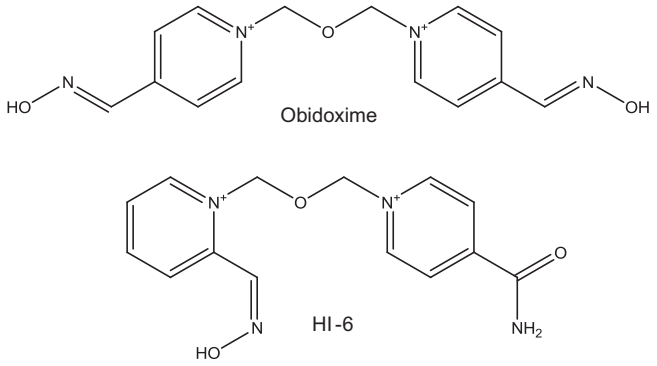


**Fig. 2.** Chemical structures of oximes.

Reactivation of OP-inhibited AChE was evaluated with HI-6 and obidoxime (Fig. 2), two widely studied oximes that have been reported to have different specificities as a result of the dissimilar positions of the oxime groups on their pyridinium rings [9]. Based on our model of OP orientation in the active site of AChE, we assumed that sarin and cyclosarin would be positioned with their methyl substituents in the acyl pocket and their alkoxy substituents in the choline pocket of AChE while the methoxy analogues of sarin and cyclosarin would be positioned with their alkyl substituents in the acyl pocket and their methoxy substituents in the choline pocket. We also assumed that our tabun analogue, in which an isosteric isopropyl group replaced the dimethylamine group of tabun, should be positioned in the active site of AChE in the same orientation as tabun. Since the other substituents were identical for AChE inhibited by tabun and its carbon analogue, this would allow us to assess the importance of the electronic effect of tabun's dimethylamine group on reactivation of tabun-inhibited AChE by comparison of its reactivation to the reactivation of AChE inhibited with a carbon analogue of tabun.

The general structure of OP-AChE conjugates is shown in Fig. 3 [14–16]. The phosphorus is covalently bonded to AChE through the active site serine residue. The phosphoryl oxygen binds in the oxyanion hole and the OP substituents designated as $R_{acyl}$ and $R_{chol}$ bind in the acyl pocket and choline pockets of AChE, respectively. These OP substituents can be amines, alkylamines, alkoxy or alkyl groups, depending on the OP compound.

The steric and electronic properties of the substituents of the parent OP agents and their analogues are shown in Tables 2 and 3, respectively. The sums of the molecular volumes of the substituents that we expected to be bound in the acyl and choline pockets varied by less than 6% (Table 2). However, the sizes of the individual substituents in the acyl pocket varied 3-fold between sarin and its analogue and 5-fold between cyclosarin and its analogue. In contrast, there was <10% difference between the sizes of the substituents in the acyl pocket for tabun and its analogue. Since we expected the electronic donation or withdrawal effect of substituents to be exerted additively on the phosphorus of OP compounds, our major interest was not the individual electronic constants of the substituents in the acyl and choline pockets, but their sums (i.e., $\sigma_{acyl} + \sigma_{chol}$), which varied <11% (Table 3).

A comparison of the ability of oximes to reactivate AChE inhibited by either sarin, cyclosarin, tabun or their analogues is shown

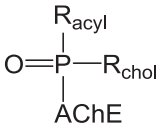


**Fig. 3.** General structure of OP-inhibited AChE.

**Table 2**
Steric properties of OP agents and analogues.

| Agents or analogue | $R_{acyl}$ substituent | $R_{chol}$ substituent | Molecular volume (Å$^3$) | | |
|---|---|---|---|---|---|
| | | | $R_{acyl}$ | $R_{chol}$ | $R_{acyl} + R_{chol}$ |
| Sarin | $CH_3$ | $OCH(CH_3)_2$ | 21.2 | 66.4 | 87.6 |
| Sarin analogue | $CH(CH_3)_2$ | $OCH_3$ | 59.0 | 28.2 | 87.2 |
| Cyclosarin | $CH_3$ | Oc-$C_6H_{11}$ | 21.2 | 108.4 | 129.6 |
| Cyclosarin analogue | c-$C_6H_{11}$ | $OCH_3$ | 100.9 | 28.2 | 129.1 |
| Tabun | $N(CH_3)_2$ | $OC_2H_5$ | 53.3 | 45.2 | 98.5 |
| Tabun analogue | $CH(CH_3)_2$ | $OC_2H_5$ | 59.0 | 45.2 | 104.2 |

**Table 3**
Electronic properties of OP agents and analogues.

| Agents or analogue | $R_{acyl}$ substituent | $R_{chol}$ substituent | Electronic constant ($\sigma^{ph}$) | | |
|---|---|---|---|---|---|
| | | | $R_{acyl}$ | $R_{chol}$ | $R_{acyl} + R_{chol}$ |
| Sarin | $CH_3$ | $OCH(CH_3)_2$ | −0.96 | −0.29 | −1.28 |
| Sarin analogue | $CH(CH_3)_2$ | $OCH_3$ | −1.30 | −0.12 | −1.42 |
| Cyclosarin | $CH_3$ | Oc-$C_6H_{11}$ | −0.96 | −0.35 | −1.31 |
| Cyclosarin analogue | c-$C_6H_{11}$ | $OCH_3$ | −1.19 | −0.12 | −1.31 |
| Tabun | $N(CH_3)_2$ | $OC_2H_5$ | −1.22 | −0.21 | −1.43 |
| Tabun analogue | $CH(CH_3)_2$ | $OC_2H_5$ | −1.30 | −0.21 | −1.51 |

in Table 4. AChE inhibited by either sarin or cylclosarin was at least 70-fold more easily reactivated by either HI-6 or obidoxime than AChE inhibited by their OP analogues. If we are correct that the methyl substituent binds in the acyl pocket for the parent OP agents while the larger alkyl group binds in the acyl pocket for the OP analogues, then these differences in reactivation are primarily the result of a steric effect. However, other mechanisms, such as aging of OP-inhibited AChE, re-inhibition of AChE by phosphylated oximes and the chirality of the OP-AChE conjugates being reactivated, may have contributed to the differences in reactivation between AChE inhibited by the parent OP agents and AChE inhibited by their OP analogues.

Resistance to reactivation of AChE inhibited by the OP analogues did not appear to result from an aging mechanism because nearly 100% reactivation of the OP analogue-AChE conjugates could be achieved if oxime reactivation was allowed to proceed for several days (Data not shown). However, the observed reactivation rates for AChE inhibited by OP analogues may have been reduced by re-inhibition of AChE by phosphylated oximes formed during the reactivation process. Phosphylated pyridinium oximes, particularly those produced by pyridinium oximes whose oxime group is para to the ring quaternary nitrogen, such as obidoxime, are sufficiently stable [17] that re-inhibition of AChE has been observed [4]. To address this potential problem our reactivation studies were conducted with 1 nM concentrations of OP-inhibited AChE, which has been shown to minimize re-inhibition of AChE by phosphylated oximes [18].

The chirality of OP compounds has a major effect on both their ability to inhibit AChE [19] and the ability of oximes to reactivate the resulting OP-AChE conjugates [20]. AChE conjugates inhibited by $S_p$ enantiomers of OP compounds are generally more easily reactivated than AChE conjugates inhibited by the corresponding $R_p$ enantiomers and this effect is enhanced as the size of the *O*-alkyl substituents of OP inhibitors increases [20]. Therefore, the stereoselective reactivation of OP-inhibited AChE might contribute to the resistance to reactivation of AChE inhibited by OP analogues if AChE exhibited a preference for inhibition by $S_p$ enantiomers of sarin and cyclosarin and by $R_p$ enantiomers of their OP analogues. However, this stereoselectivity hypothesis cannot be tested until we isolate the resolved enantiomers of the OP analogues.

**Table 4**
Comparison of oxime reactivation of AChE inhibited by OP agents and analogues.

| Agents or analogue | $R_{acyl}$ substituent | $R_{chol}$ substituent | AChE reactivation rate constant ($mM^{-1}$ $min^{-1}$)[a] | |
|---|---|---|---|---|
| | | | HI-6 | Obidoxime |
| Sarin | $CH_3$ | $OCH(CH_3)_2$ | 13.9 ± 3.7 | 15.6 ± 4.7 |
| Sarin analogue | $CH(CH_3)_2$ | $OCH_3$ | 0.085 ± 0.010 | 0.13 ± 0.04 |
| Cyclosarin | $CH_3$ | Oc-$C_6H_{11}$ | 17.0 ± 5.2 | 0.43 ± 0.11 |
| Cyclosarin analogue | c-$C_6H_{11}$ | $OCH_3$ | 0.003 ± 0.001 | 0.006 ± 0.002 |
| Tabun | $N(CH_3)_2$ | $OC_2H_5$ | NR[b] | 0.21 ± 0.06 |
| Tabun analogue | $CH(CH_3)_2$ | $OC_2H_5$ | 0.061 ± 0.023 | 0.10 ± 0.03 |

[a] Rate constants are presented as means ± standard errors.
[b] No reactivation was observed within 4 h at 1 mM concentration of oxime.

**Table 5**
Steric effect of OP acyl substituent on oxime reactivation of OP-inhibited AChE.

| Agents or analogue | $R_{acyl}$ substituent | $R_{chol}$ substituent | Molecular volume of $R_{acyl}$ ($Å^3$) | AChE reactivation rate constant ($mM^{-1}$ $min^{-1}$)[a] | |
|---|---|---|---|---|---|
| | | | | HI-6 | Obidoxime |
| Methamidophos | $NH_2$ | $OCH_3$ | 12.4 | 14.3 ± 3.1 | 25.4 ± 5.7 |
| Methyl sarin | $CH_3$ | $OCH_3$ | 21.2 | 12.1 ± 4.6 | 22.1 ± 5.1 |
| VX | $CH_3$ | $OC_2H_5$ | 21.2 | 13.5 ± 3.7 | 20.1 ± 4.0 |
| Methyl paraoxon | $OCH_3$ | $OCH_3$ | 28.2 | 0.69 ± 0.21 | 24.3 ± 4.2 |
| Paraoxon | $OC_2H_5$ | $OC_2H_5$ | 45.2 | 0.45 ± 0.14 | 21.5 ± 3.0 |
| Tabun | $N(CH_3)_2$ | $OC_2H_5$ | 49.1 | NR[b] | 0.21 ± 0.06 |
| Sarin analogue | $CH(CH_3)_2$ | $OCH_3$ | 59.0 | 0.085 ± 0.010 | 0.13 ± 0.04 |
| Tabun analogue | $CH(CH_3)_2$ | $OC_2H_5$ | 59.0 | 0.061 ± 0.023 | 0.10 ± 0.03 |
| Cyclosarin analogue | c-$C_6H_{11}$ | $OCH_3$ | 100.9 | 0.003 ± 0.001 | 0.006 ± 0.002 |

[a] Rate constants are presented as means ± standard errors.
[b] No reactivation was observed within 4 h at 1 mM concentration of oxime.

In contrast to the large differences in oxime reactivation that we observed between AChE conjugates of sarin or cyclosarin and their analogues, the difference in obidoxime reactivation between AChE conjugates of tabun and its isosteric carbon analogue was only 2-fold. This suggested that the effect of tabun's dimethylamine group that results in resistance to oxime reactivation of tabun-inhibited AChE was primarily steric and not electronic. However, this conclusion was tempered by the absence of any reactivation of tabun-inhibited AChE by HI-6, because HI-6 was able to reactivate AChE inhibited by the tabun analogue. Although the steric effect of acyl OP substituents appeared to be the dominant mechanism to explain the resistance of OP-inhibited AChE to reactivation, the difference between HI-6 reactivation of the AChE-tabun conjugate and HI-6 reactivation of the AChE-tabun analogue conjugate suggested that the steric effect of acyl OP substituents is not the entire explanation.

In order to put our studies of oxime reactivation of AChE inhibited by sarin, cyclosarin, tabun and their analogues into a larger perspective, we also conducted an oxime reactivation study with AChE that had been inhibited with additional OP compounds whose acyl substituents encompassed a wider range of molecular volumes (Table 5). The OP compounds in this study consisted of OP inhibitors with acyl substituents whose molecular volumes varied 8-fold. This study demonstrated that the pattern of the steric effect exerted by the putative acyl substituents on oxime reactivation of OP-inhibited AChE was initially neutral followed by a progressively more severe negative effect, which occurred whether the OP inhibitor was a phosphate, phosphonate or phosphoramidate. However, the transition from neutral effect to negative effect on reactivation as the size of the acyl substituent increased was dependent on the oxime. For HI-6 reactivation of OP-inhibited AChE the onset of the negative steric effect on reactivation occurred when the volume of the acyl substituent increased from a methyl group to a methoxy group. For obidoxime the onset of this negative steric effect on reactivation occurred when the volume of the acyl substituent increased from an ethoxy group to a dimethylamine group. An understanding at the molecular level for obidoxime's reduced sensitivity to the steric effect of acyl substituents on reactivation may provide a basis for designing improved reactivators in the future. Nevertheless, at the present time the significance of our report is that it identified a common mechanism for resistance to reactivation of OP-inhibited AChE that is applicable to a structurally diverse range of OP inhibitors.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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